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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
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| 08/765,695 | 07/25/1997 | LARS ABRAHMSEN | A96335US | 6468 |

7590 06/16/2004
FULBRIGHT & JAWORSKI LLP
1301 MCKINNEY
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HOUSTON, TX 77010-3095

EXAMINER

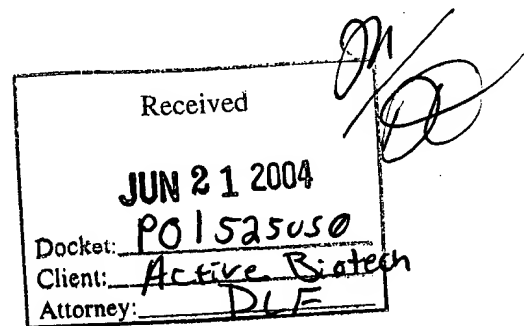
SCHWADRON, RONALD B

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| ART UNIT | PAPER NUMBER |
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1644

DATE MAILED: 06/16/2004

Please find below and/or attached an Office communication concerning this application or proceeding.





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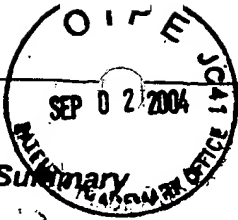
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| EXAMINER | |
| SCHWADRON, RONALD B | |
| ART UNIT | PAPER NUMBER |
| 1644 | |

DATE MAILED: 05/26/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary



Application No.

08/765,695

Applicant(s)

ABRAHMSSEN ET AL.

Examiner

Ron Schwadron, Ph.D.

Art Unit

1644

- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --
Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☐ Responsive to communication(s) filed on ____.
- 2a) ☐ This action is FINAL. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 36,58-63 and 65-70 is/are pending in the application.
- 4a) Of the above claim(s) ____ is/are withdrawn from consideration.
- 5) ☒ Claim(s) 70 is/are allowed.
- 6) ☒ Claim(s) 36,58-63,65-68 is/are rejected.
- 7) ☒ Claim(s) 69 is/are objected to.
- 8) ☐ Claim(s) ____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on ____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☒ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. ____.
 3. ☒ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
 Paper No(s)/Mail Date ____.
- 4) ☐ Interview Summary (PTO-413)
 Paper No(s)/Mail Date ____.
- 5) ☐ Notice of Informal Patent Application (PTO-152)
- 6) ☐ Other: ____.

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 3/9/2004 has been entered.

2. The previously pending rejections are withdrawn in view of the amended claims.

3. The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

4. Claims 36,58-63,65-68 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for the claimed method that uses the D227A mutant, does not reasonably provide enablement for the method that uses combinations of the amino acid substitutions recited in the claims or amino acid substitutions other than D227A. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims.

The specification is not enabling for the claimed method that uses combinations of the amino acid substitutions recited in the claims or amino acid substitutions other than D227A. Applicant has not enabled the breadth of the claimed invention in view of the teachings of the specification because the use for the instant invention disclosed in the specification is the treatment of disease in vivo. The state of the art is such that is unpredictable in the absence of appropriate evidence as to how the instant invention could be used for treating disease using combinations of the amino acid substitutions recited in the claims or amino acid substitutions other than D227A.

Judge Lourie stated in Enzo Biochem Inc. v. Calgene Inc. CAFC 52 USPQ2d 1129 that:

The statutory basis for the enablement requirement is found in Section 112, Para. 1, which provides in relevant part that:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same. . . .

35 U.S.C. Section 112, Para. 1 (1994). "To be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation.'" Genentech, Inc. v. Novo Nordisk, A/S , 108 F.3d 1361, 1365, 42 USPQ2d 1001, 1004 (Fed. Cir. 1997) (quoting In re Wright , 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993)). Whether claims are sufficiently enabled by a disclosure in a specification is determined as of the date that the patent application was first filed, see Hybritech, Inc. v. Monoclonal Antibodies, Inc. , 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), which in this case is October 20, 1983 for both the '931 and '149 patents.

We have held that a patent specification complies with the statute even if a "reasonable" amount of routine experimentation is required in order to practice a claimed invention, but that such experimentation must not be "undue." See, e.g., Wands , 858 F.2d at 736-37, 8 USPQ2d at 1404 ("Enablement is not precluded by the necessity for some experimentation However, experimentation needed to practice the invention must not be undue experimentation. The key word is 'undue,' not 'experimentation.'" (footnotes, citations, and internal quotation marks omitted). In In re Wands , we set forth a number of factors which a court may consider in determining whether a disclosure would require undue experimentation. These factors were set forth as follows:

(1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

Id. at 737, 8 USPQ2d at 1404. We have also noted that all of the factors need not be reviewed when determining whether a disclosure is enabling. See Amgen, Inc. v. Chugai Pharm. Co., Ltd. , 927 F.2d 1200, 1213, 18 USPQ2d 1016, 1027 (Fed. Cir.

1991) (noting that the Wands factors "are illustrative, not mandatory. What is relevant depends on the facts.").

Regarding Wands factor (3), there are no working examples in the specification using the F47A, N128A, H187A or H225A SEA mutants in the claimed method or examples using combinations of the mutations recited in claim 36 in the claimed method. Regarding Wands factors (4) and (8), the claims encompass treatment of disease in vivo. Regarding Wands factors (5) and (7), there is a high degree of unpredictability in the art. For example, Forsberg et al. disclose that while a SEA mutant containing D227A was used to treat tumors in a SCID mouse model (see abstract), a mutant containing a F47A/D227A was not used because it did not appear that such a mutant would work ("However, binding to MHC class II is important to obtain inflammatory cytokines and to activate T-cells. Therefore, the second MHC class II binding site surrounding Phe 47 was not altered.")(see page 135, second column, first complete paragraph). Thus, it is unclear whether a mutant using combinations of the substitutions recited in claims could be used to treat disease in vivo. Furthermore, regarding any particular substitution other than D227A, as stated above, a certain degree of MHC class II binding needs to be found in order to obtain inflammatory cytokines and to activate T cells. For example, Figure 4 of Forsberg et al. shows that the D227A SEA mutant has a particular effect on cytokine secretion in vivo. There is no evidence of record that the other mutants recited in the claims would have similar effects. In addition, Newton et al. discloses that SEA D227A activates a specific subset of VB whilst SEA F47A activates a different subset of VB. Thus, it is unclear whether SEA F47A would activate T cells in vivo that are activated by SEA D227A and therefore it is unclear whether other SEA mutants would activate T cells required to treat disease. Based on the aforementioned undue experimentation would be required of one skilled in the art to practice the instant invention using the teaching of the specification.

5. Claim 69 is objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

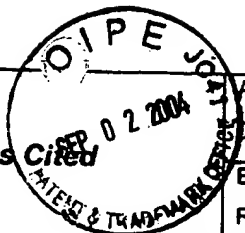
6. Claim 70 is allowed.

7. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ron Schwadron, Ph.D. whose telephone number is 571 272-0851. The examiner can normally be reached Monday to Thursday from 7:30am to 6:00pm. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan, can be reached on 571 272 0841. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306. Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Ron Schwadron, Ph.D.
Primary Examiner
Art Unit 1644


RONALD B. SCHWADRON
PRIMARY EXAMINER
GROUP 1800 1644

Notice of References Cited



Application/Control No.

08/765,695

Applicant(s)/Patent Under Reexamination
ABRAHMSSEN ET AL.

Examiner

Ron Schwadron, Ph.D.

Art Unit

1644

Page 1 of 1

U.S. PATENT DOCUMENTS

| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Name | Classification |
|---|---|--|-----------------|------|----------------|
| | A | US- | | | |
| | B | US- | | | |
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| | G | US- | | | |
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| | I | US- | | | |
| | J | US- | | | |
| | K | US- | | | |
| | L | US- | | | |
| | M | US- | | | |

FOREIGN PATENT DOCUMENTS

| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Country | Name | Classification |
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NON-PATENT DOCUMENTS

| * | | Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages) |
|---|---|---|
| | U | Forsberg et al., Brit. J. Cancer, 85:129-136, 2001. |
| | V | Journal Newton et al., The Journal of Immunol., 157:3988-3994, 1996. |
| | W | |
| | X | |

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

U.S. Patent and Trademark Office
PTO-892 (Rev. 01-2001)

Notice of References Cited

Part of Paper No. 200405

RLJ

5/25/2004

Therapy of human non-small-cell lung carcinoma using antibody targeting of a modified superantigen

G Forsberg¹, L Ohlsson¹, T Brodin¹, P Björk¹, PA Lando¹, D Shaw², PL Stern² and M Dohlsten¹

¹Active Biotech Research AB, Box 724, 220 07 Lund, Sweden; ²Paterson Institute for Cancer Research, Manchester, UK;
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Summary Superantigens activate T-cells by linking the T-cell receptor to MHC class II on antigen-presenting cells, and novel reactivity can be introduced by fusing the superantigen to a targeting molecule. Thus, an antibody-targeted superantigen, which activates T cells to destroy tumour cells, might be used as cancer therapy. A suitable target is the 5T4 oncofetal antigen, which is expressed on many carcinomas. We constructed a fusion protein from a Fab of a monoclonal antibody recognizing the 5T4 antigen, and an engineered superantigen. The recombinant product 5T4FabV13-SEA_{D227A} bound the 5T4 antigen expressed on the human non-small-cell lung cancer cell line Calu-1 with a K_D of 1.2 nM while the substitution of Asp227 to Ala in the superantigen moiety reduced binding activity to MHC class II. 5T4FabV13-SEA_{D227A} tumour reactivity was demonstrated in 7/7 NSCLC samples by immunohistochemistry, while normal tissue reactivity was low to moderate. 5T4FabV13-SEA_{D227A} induced significant T-cell-dependent *in vitro* killing of sensitive 5T4 bearing Calu-1 cells, with maximum lysis at 10^{-10} M, while the capacity to lyse MHC class II expressing cells was approximately 1000 times less effective. Immunotherapy of 5T4FabV13-SEA_{D227A} against human NSCLC was investigated in SCID mice reconstituted with human peripheral blood mononuclear cells. Mice carrying intraperitoneally growing Calu-1 cells showed significant reduction in tumour mass and number after intravenous therapy with 5T4FabV13-SEA_{D227A}. Thus, 5T4FabV13-SEA_{D227A} has highly attractive properties for therapy of human NSCLC. © 2001 Cancer Research Campaign <http://www.bjcancer.com>

Antibody-based therapies are currently evaluated for treatment of several severe diseases, such as cancer, viral infections and autoimmunity. Recent technological improvements have made it possible to clone and produce large amounts of intact recombinant monoclonal antibodies or antibody fragments with unique specificities (reviewed by Winter and Milstein, 1991; Dall'Acqua and Carter, 1998; Hudson, 1998). Several tumour-associated antigens have been identified and are currently being investigated as therapeutic targets for haematological and solid tumours (Riethmüller et al, 1993). non-small-cell lung cancer (NSCLC) is an aggressive solid tumour associated with a poor prognosis since surgery or chemotherapy is only beneficial in a fraction of all cases. Antibody-based therapy has been of limited success, but has been described using murine antibodies against targets such as the EGF-receptor in squamous cell carcinoma (Modjtahedi et al, 1996) or epithelial glycoprotein-2 for adenocarcinoma (Zimmermann et al, 1997). Such monoclonal antibodies probably may act by interfering in tumour cell signalling or through activation of complement and/or Fc receptor bearing cells. To potentiate the effects of monoclonal antibodies, the use of fusions with superantigens, which are of bacterial or viral origin and activate T cells by linking the T cell receptor to MHC class II on antigen presenting cells (reviewed by Johnson et al, 1992), have

previously been described by us (Dohlsten et al, 1994; Brodin et al, 1998). Thereby, large amounts of cytotoxic and cytokine producing T-cells can be targeted to destroy and initiate a powerful T cell attack against tumour cells *in vivo* (Dohlsten et al, 1994, 1995a). Most studies of antibody targeted superantigens, e.g. staphylococcal enterotoxin A, SEA, have been for the treatment of human colorectal cancer (Dohlsten et al, 1995b). However, to decrease the reactivity of the superantigen with MHC class II-bearing cells, the Asp227Ala replacement were introduced to destroy the site having the highest affinity for MHC class II in SEA (Abrahamsén et al, 1995).

The human 5T4 antigen was discovered by looking for shared surface molecules which would reflect the functional similarities between the growth and invasive properties of trophoblast, the major interfacing cell type between mother and fetus in the placenta, and tumour cells. The murine antibody 5T4 recognizes a 72 kDa glycoprotein (Hole and Stern, 1988, 1990) found in many carcinomas, especially non-small-cell lung and breast cancer, but at low levels in normal tissues (Southall et al, 1990). 5T4 tumour-associated labelling is also a marker of prognostic significance in colorectal (Starzynska et al, 1992, 1994, Mulder et al, 1997) and gastric carcinoma (Starzynska et al, 1998). The cDNA encoding human 5T4 predicts a heavily glycosylated membrane-bound protein with regions containing leucine rich repeats, which probably contribute directly to protein-protein interactions (Myers et al, 1994). Overexpression of 5T4 antigen alters cell adhesion, shape and motility *in vitro* (Carsberg et al, 1995, 1996). Minimal residual disease is likely to be 5T4 positive and is thus a potential candidate for superantigen mediated therapy. Here, the potential for a fusion protein between 5T4 and SEA_{D227A} was investigated for therapy of NSCLC.

Received 8 June 2000

Received 8 November 2000

Accepted 17 November 2000

Correspondence to: G Forsberg

MATERIALS AND METHODS

Cells

The 5T4 hybridoma was grown and the mAb purified as previously described (Hole and Stern, 1990). The human leukaemia cell line K562, colon adenocarcinoma WiDR, HT29, NSCLC Calu-1, ME 180 and the B cell lymphoblastoid Raji were obtained from American Type Culture Collection (Rockville, MD). All cells were mycoplasma free and maintained under standard conditions.

Peripheral blood mononuclear cells, PBMC, were from heparinized blood from normal donors at the University Hospital of Lund. The cells were isolated by density centrifugation over Ficoll-Paque cushion and incubated in complete R-medium (RPMI-1640 supplemented with 10% fetal calf serum (Gibco BRL, Life Technologies, UK), 1 mM glutamine, 10 mM Hepes buffer, 1 mM sodium pyruvate (HyClone Europe, UK), 50 μ M 2-mercaptoethanol (ICN Biomedicals INC, Costa Mesa CA), 0.1 M NaHCO₃ (Seromed Biochrome), 0.1 mg ml⁻¹ gentamycin (Biological Industries, Kibbutz Beit Haemek, Israel). SEA activated T cell lines were produced by activation of PBMC using 2×10^6 cells ml⁻¹ with mitomycin C-treated BSM cells preincubated with 100 ng ml⁻¹ SEA in medium with 10% FCS (Dohlsten et al, 1991). The T cell lines were restimulated biweekly with 20 U ml⁻¹ IL-2, weekly with mitomycin C treated SEA coated BSM cells (Van De Griend et al, 1984) and cultivated for 4–12 weeks before being used in the assay. The viability of the effector cells, as determined by trypan blue exclusion, exceeded 50%. Flow cytometric analysis and sorting were performed according to standard setting on a FACStar[™] (Becton Dickinson, Mountain View, CA, USA).

Cloning, expression and purification of Fab-SEA fusion proteins

The fusion proteins were produced at Pharmacia & Upjohn (Stockholm, Sweden) essentially as described (Dohlsten et al, 1994; Abrahmsén et al, 1995; Forsberg et al, 1997). The Fv-encoding portions of 5T4 were cloned from the 5T4 hybridoma and fused to sequences coding for the constant regions of the murine IgG1/k antibody C242 lacking the interchain disulphide bond. A region coding for SEA_{D227A} was connected to the C-terminus of the heavy chain (Figure 1A) via a Gly-Gly-Pro linker. The products were expressed and secreted in the *E. coli* K-12 strain UL 635 (*xyt-7*, *ara-14*, *T4^R*, *ΔompT*) (Abrahmsén et al, 1995) using a plasmid with a lacUV5-promoter. After fermentation, clarified growth medium was applied to a Protein G Sepharose column (Pharmacia Biotech, Uppsala, Sweden) and bound protein eluted with 0.1 M acetic acid, 0.05% Tween 80. Full-length product was separated from a degraded variant lacking the superantigen moiety, 5T4FabV13, on an SP Sepharose HP column (Pharmacia Biotech) using a linear gradient from 60 to 350 mM sodium acetate (Forsberg et al, 1997). SDS-PAGE and chromatographic techniques indicate that the purity of the product was at least 95%.

Assays

To measure lymphocyte proliferation by incorporation of [³H]-thymidine, 2×10^5 PBMC were incubated at 37°C in 200 μ l complete R-medium with titrating amounts of Fab-SEA proteins for 72 h, as described (Dohlsten et al, 1988). Tumour cell growth-inhibition

assay were performed using 5×10^3 tumour cells well in 96-well flat-bottomed microtitre wells (Nunc, Roskilde, Denmark) in complete R-medium and titrating dilutions of supernatants from PBMC incubated for 72 h with 10^{-9} M of Fab-SEA fusion proteins in a total volume of 200 μ l. Tumour cells were then incubated for 72 h and the viable fraction of cells determined using the MTT-assay (Van de Loosdrecht et al, 1991). The production of IL-2, IFN- γ and TNF- α in 2×10^6 PBMC ml⁻¹ incubated at 37°C with titrating amounts of Fab-SEA proteins, were measured in culture supernatants from plates in 2 ml R-medium with specific ELISA reagents (Genzyme Corporation, Cambridge, MA) as recommended by the supplier.

To study cytotoxicity, K562, Calu-1 or Raji cells, labelled with (Na)₂⁵¹CrO₄ (Amersham Solna, Sweden) were used in a standard 4 h chromium release assay (Dohlsten et al, 1994). As effector cells, an SEA reactive T cell line, prepared as described above, was used. This cell line contains more than 99% CD3-positive cells (Hedlund et al, 1990). The specific cytotoxicity was calculated using the average counts/min (cpm) in the formula: specific cytotoxicity = (experimental cpm – spontaneous release cpm) / (total release cpm – spontaneous release cpm).

Binding assays

Affinities to the 5T4 antigen were measured similarly to Forsberg et al (1997). 5T4FabV13-SEA_{D227A} and 5T4FabV13 respectively were labelled with Na¹²⁵I (NEN, Boston, MA) to obtain a specific activity of 10 to 40 μ Ci μ g⁻¹ protein and an iodine to protein ratio of $\leq 2:1$. Serially diluted ¹²⁵I-labelled 5T4FabV13-SEA_{D227A} or 5T4FabV13 in triplicate was incubated with Calu-1 or ME 180 cells for 2 h at room temperature. After washing, cell-bound radioactivity was determined. The dissociation constant, *K_d*, and number of binding sites, *N*, at saturation were calculated (Scatchard, 1949) after subtraction of non-specific binding. To determine affinities to MHC class II, plasma membranes were prepared from Raji cells (Massague, 1987). Approximately 10^8 frozen cells were homogenized in 10 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, pH 7, containing 0.25 M sucrose with 20 strokes in a pre-cooled Dounce homogenizer and centrifuged 10 min at 6000 rpm. The pellet was resuspended in that buffer with sucrose and centrifuged. The combined supernatants were layered on top of a cushion containing 37% w/v sucrose in buffer and centrifuged at 105 000 $g \times 60$ min. The membrane layer was removed, diluted 4 times with buffer and centrifuged at 30 000 $g \times 30$ min. The pellet was resuspended in 1 ml buffer and stored at -70 °C. Plasma membranes were immobilised as described by Vater et al (1995). Aliquots from fractionated plasma membranes were diluted in 5 mM Na₂S₂O₃ and 1 mM PMSF and distributed on a 96-well Immulon 2 ELISA plate (Dynatech Labs). The plates were dried, unbound sites blocked for 1 h and washed twice in Tris-buffered saline containing 3% defatted milk. Serially diluted biotinylated SEA (long-arm NHS-biotin reagent, Vector Labs) was incubated with 5T4FabV13-SEA_{D227A}, SEA or C242Fab-SEA in blocking buffer for 5 min and then membranes for 2 h at room temperature. Detection was carried out using the Vectastain® ABC Kit (BioRad).

Immunohistochemistry

7 histologically characterized samples of NSCLC (5 adenocarcinoma and 2 squamous cell carcinoma) were from Dr Jayant Shetye

(Department of Pathology, Karolinska Hospital). Normal tissues (see Table 1) and breast tumours were provided by the Department of Surgery, Lund University Hospital. Acetone fixed cryosections of the above tissues were labelled with 5T4FabV13SEA_{D227A} followed by biotinylated rabbit anti-SEA (in house production). After incubation in streptavidin-biotin/horseradish peroxidase (Dako, Copenhagen) the sections were developed in diaminobenzidine (Savcen AB, Malmö), counterstained in methyl green and mounted. As reference to reaction seen in vessels anti human CD31 antibody was used (Dako, Copenhagen). As negative controls irrelevant FabSEA_{D227A} and mouse monoclonal IgG1 were used.

In vivo induction of cytokines

All animal were kept under pathogen-free conditions and the experiments carried out using approved ethical protocols. C57/B16 mice got 4 daily intravenous injections of 30 µg control Fab-SEA or 5T4FabV13-SEA_{D227A} in PBS or buffer alone. Blood samples were taken by caval vein puncture at various time points. All groups contained pooled sera from 3 animals. The levels of IL-2, IL-6, TNF-α and IFN-γ were measured as above.

Therapy in SCID mice

Severe Combined Immunodeficient (SCID) female mice (C.B-17, Bommeice, Ry, Denmark) were injected intraperitoneally with 3×10^6 Calu-1 cells in vehicle (0.2 ml PBS-1% Balb/c mouse serum) and 5 days later I.P. with 3×10^6 PBMC in 0.2 ml vehicle. 1 to 2 h after injection of PBMC all mice were injected intravenously with 5T4FabV13-SEA_{D227A} or the non-binding control C215Fab-SEA_{D227A} (Hansson et al, 1997) in 0.2 ml vehicle or vehicle alone. 2 additional intravenous injections of the respective test substance were given with 3 day intervals unless otherwise specified. The mice were sacrificed between day 30 to 40, by cervical dislocation and the number of tumours and the tumour mass determined. Tumours of less than 5 mg were estimated as 2 mg, tumours with a mass of more than 5 mg and less than 10 mg as 7 mg and tumours larger than 10 mg with the actual weight. All tumours larger than 1 mg were counted. Each treatment cohort contained 5 to 7 mice to

permit comparison to other treatment cohorts treated simultaneously with the same batch of effector cells. Statistical significance was determined by the Mann-Whitney U test. For histochemical analysis, 6 µm cryosections were analysed as above.

RESULTS

E. coli production of the fusion protein

The fusion protein 5T4FabV13-SEA_{D227A} (Figure 1A) was produced as a secreted product in *E. coli*. The product was expressed as bicistronic construct with SEA_{D227A} fused to the C-terminus of the Fab heavy chain. The production level was increased 15-fold by replacing 7 amino acid residues in the framework of 5T4Fab, yielding 5T4FabV13 (Forsberg et al, 1997). The yield in the *E. coli* growth medium of 5T4FabV13-SEA_{D227A} was in the order of 0.5 g l⁻¹. To decrease the MHC class II binding, and subsequently in vivo toxicity, the replacement Asp227Ala was introduced in SEA, yielding SEA_{D227A} (Abrahmsén et al, 1995). The full-length product was recovered in a 2-step purification procedure. The purity of the product was at least 95% as determined by SDS-PAGE and chromatographic techniques. In addition to the full-length product, a truncated variant corresponding to 5T4V13Fab, was recovered in the second purification step.

Tissue reactivity of 5T4FabSEA_{D227A}

The expression of 5T4 antigen in tumour and normal tissue was investigated with immunohistochemistry. 5T4FabV13-SEA_{D227A}

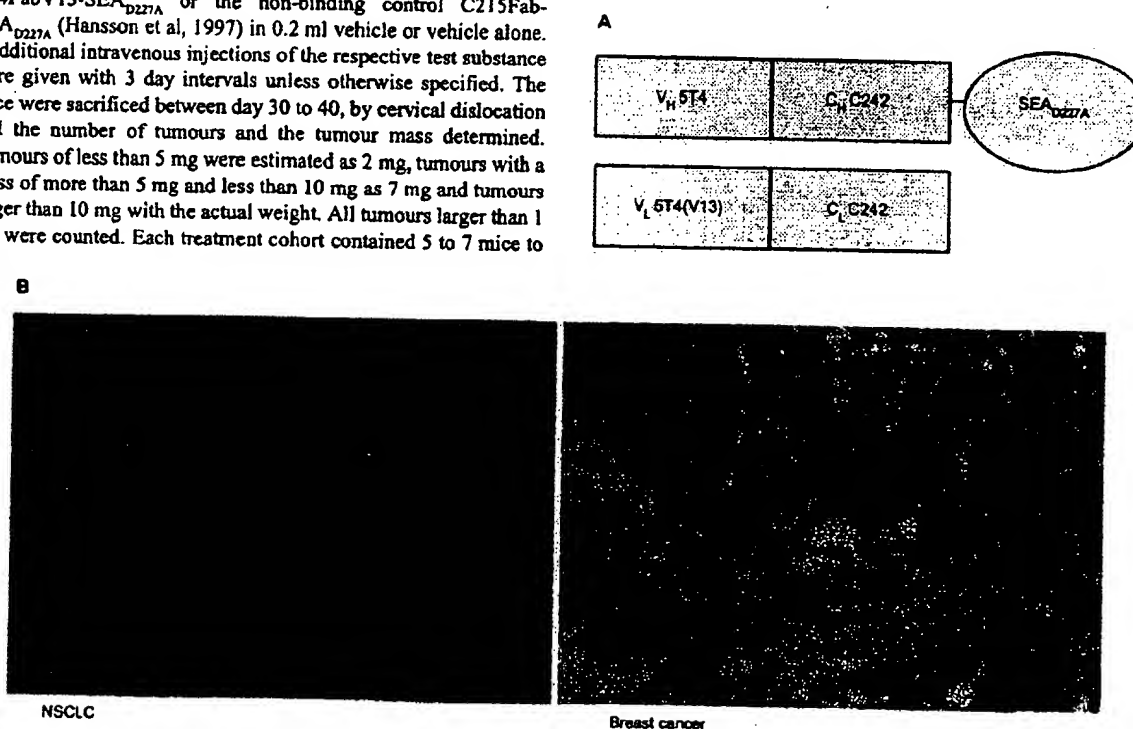


Figure 1 (A) Schematic representation of 5T4FabV13-SEA_{D227A}. The product consists of a modified variant of the variable regions from 5T4 antibody (Forsberg et al, 1997) connected to the CL and CH1 domains of the antibody C242 (Dohlsien et al, 1994). The superantigen SEA_{D227A} is fused to the C-terminus of CH1. (B) Immunohistochemical analysis of the 5T4-antigen expression in NSCLC tumour tissue and breast cancer tissue. Strong expression is observed on both the tumour cells (T) as well as in the stroma (S).

Table 1 Summary of the immunohistochemical analysis of NSCLC, breast cancer and normal tissue. The tissue-reactivities were scored semiquantitatively according to the intensity of the staining. A + equals weak reaction, ++ moderate reaction and +++ strong reaction

| Tissue | Reactivity | Comment |
|--|------------|---|
| NSCLC (n = 7) | ++ -+++ | Homogenous staining in 7/7 samples, 5 adenocarc. and 2 squamous carc. |
| Breast ca. (n = 6) | ++ -+++ | Homogenous staining in 6/6 samples. |
| CNS (n = 1) | neg. | |
| Skin (n = 2) | neg. | |
| Myocardium (n = 4) | + | Reaction in the luminal outline of a subpopulation of muscular vessels in 2/4 samples. |
| Adrenal (n = 2) | neg. | |
| Kidney (n = 4) | + -++ | Weak-moderate diffuse reaction in glomeruli and parietal layer outlining Bowman's capsule (2/4). Weak focal reaction outlining lumen in occasional muscular vessels (2/4). |
| Lung (n = 4) | + -++ | Weak luminal outline in occasional vessels (2/4). Moderate reaction in a basal epithelial cellular or matrix component associated with the bronchial epithelium. |
| Liver (n = 4) | + | Occasional staining of the sinusoidal outline close to the central vein (1/4). |
| Pancreas (n = 2) | + -++ | Weak-moderate reaction in occasional pancreatic ducts and scarce stroma structures. Weak focal reaction outlining lumen in occasional muscular vessels. |
| Gastro-intestinal tract (stomach n = 2, small intestine n = 2 and large intestine n = 4) | ++ | Reaction in some cell type or extracellular component of the epithelial basal lamina or the lamina propria in parts of the surface epithelium |
| Pharynx (n = 2) | + -++ | Reaction in squamous epithelium (most prominent in basal layer). |
| Thyroid (n = 2) | + | Reaction associated with follicular epithelial cells. Focal reaction outlining lumen of occasional muscular vessels. |
| Spleen (n = 2) | + | Focal reaction outlining lumen of occasional muscular vessels. |

tumour reactivity was demonstrated in 7/7 cases of NSCLC, including 5 adenocarcinomas and 2 squamous cell carcinomas (Table 1). Moderate staining was seen in 4 of the adenocarcinomas and 1 squamous cell carcinoma and moderate to strong in the remaining. In a group of 6 breast carcinomas, moderate reactions were seen in 5 of them and a moderate-strong in the sixth. Reaction was not only confined to the tumour cells since all examined tumours (both NSCLC and breast carcinomas) also showed stromal reactivity (Figure 1B). In some cases the stromal reaction dominated over the tumour cell reaction. 5T4FabV13-SEA_{D227A} reactivity was also assessed in some normal tissues (Table 1) and was found to be similar to that seen with the mAb (Southall et al, 1990). This reactivity was not observed with an irrelevant FabSEA_{D227A} protein. The normal tissue reactivity is presented in Table 1. The most quantitatively dominating normal tissue reactivity was found in some cell type or extra-cellular component found in association with the basal membrane or the lamina propria of the alimentary tract. Focally weak reaction was seen in the luminal outline of a minority (less than 10%) of muscular blood vessels in different normal tissues. This reaction showed individual variation since only 2/4 colon samples, 2/4 lung and 2/4 myocardial samples demonstrates it.

In kidney 2/4 showed weak diffuse, possibly intracellular reaction in glomeruli and outlining of the parietal layer of Bowman's capsule while the other 2 showed weak-moderate reaction in these structures. In the liver 1/3 samples showed a weak staining outlining sinusoids proximal to the central vein. Weak-moderate reaction was also found in duct epithelium of pancreas and in squamous epithelium of pharynx and weak reaction was found in the epidermal layer of the skin and in association with the follicular epithelium of the thyroid gland.

Binding affinity of 5T4FabV13-SEA_{D227A} to the 5T4 antigen and MHC class II

Several cell lines were investigated for 5T4 antigen expression using flow cytometry and the strongest 5T4 FACS positive NSCLC line, Calu-1, was used to measure the affinity of the fusion protein to the 5T4 antigen. In FACS analysis, the fusion protein 5T4FabV13-SEA_{D227A} bound to the cells in a dose-dependent manner with maximum binding at 10^{-4} M (data not shown). Radioiodinated 5T4FabV13-SEA_{D227A} and 5T4FabV13 were used for the antigen-binding assays. Figure 2A shows that both reagents have nM affinity (mean K_D of 1.2×10^{-9} M or 2.3×10^{-9} M respectively) with an antigen density of approximately 3×10^5 molecules per cell. Binding of 5T4FabV13-SEA_{D227A} was also measured on ME 180 cells. Here, the affinity was slightly higher, 0.7×10^{-9} M, while the number of binding sites were approximately 1.3×10^5 molecules per cell (data not shown).

The binding to MHC class II expressed on Raji cells was then investigated. In accordance with previous findings (Dohlsten et al, 1994), Fab-SEA was a much weaker competitor for binding to MHC class II molecules than SEA. SEA and Fab-SEA showed IC_{50} values of approximately 21 and 360 nM respectively in a competitive assay with biotinylated SEA to coated Raji cell plasma membranes. 5T4FabV13-SEA_{D227A} did not show any displacement in the concentration range used ($\leq 20 \mu\text{M}$) and thus has an affinity of less than $10 \mu\text{M}$ (Figure 2B).

Cytotoxicity of 5T4FabV13-SEA_{D227A} to NSCLC and MHC class II-expressing cells

To investigate T-cell mediated cytotoxicity on tumour cells induced by the fusion protein in vitro, 5T4FabV13-SEA_{D227A} was

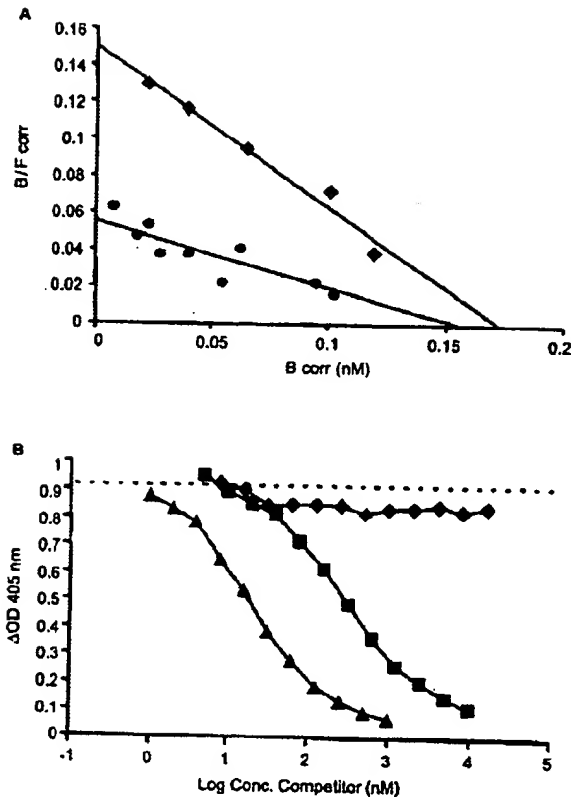


Figure 2 (A) Representative Scatchard plot and saturation curve for binding of ^{125}I -5T4FabV13-SEA_{D227A} (♦) and ^{125}I -5T4FabV13 (●) to Calu-1 cells. Values are corrected for non-specific binding. The calculated K_d in this experiment were 1.2 and 2.3 nM respectively with 300 000 sites/cell. Each point represents the mean of triplicates. (B) Binding of 10 nM biotinylated SEA to immobilized MHC class II positive Raji cell plasma membranes in the presence of SEA (▲), C242Fab-SEA (Dohlstien et al, 1995b) (■) or 5T4FabV13-SEA_{D227A} (♦) as competitors. Each point represents the mean of duplicates

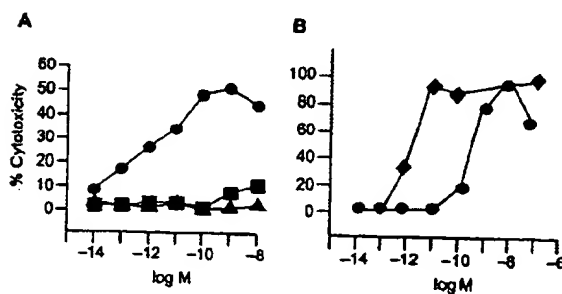


Figure 3 Induced cytotoxicity using 5T4FabV13-SEA_{D227A} on (A) chromium-labelled 5T4 expressing Calu-1 cells and (B) chromium-labelled MHC class II-expressing Raji cells. The symbol ● represents 5T4FabV13-SEA_{D227A}, ▲ control Fab-SEA_{D227A}, □ 5T4FabV13-SEA_{D227A} in the absence of T cells and control Fab-SEA. The effects were mediated by fusion proteins and human activated T cells. The cells were incubated for 4 h, then the released chromium was measured and the % cytotoxicity determined. 5T4FabV13-SEA_{D227A} was only active in the presence of T cells and at least 100-fold more potent against Calu-1 cells

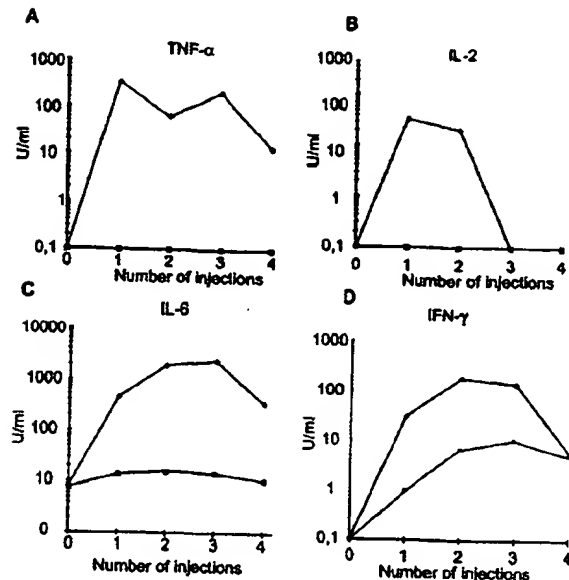


Figure 4 Serum cytokine levels after injection of C215Fab-SEA (♦) (Dohlstien et al, 1994) and 5T4FabV13-SEA_{D227A} (■) in C57/B16 mice. The use of the mutated superantigen significantly reduced the systemic release of (A) TNF-α, (B) IL-2, (C) IL-6 and (D) IFN-γ. A 10- to 100-fold reduction in the serum levels of IL-2 and IFN-γ and a 100 to 1000 reduced amount of TNF-α and IL-6 was recorded in animals treated with SEA_{D227A} containing fusion protein

mixed with chromium labelled Calu-1 cells and human SEA reactive T cells (Dohlstien et al, 1991). 5T4FabV13-SEA_{D227A} mediated a specific T cell killing of Calu-1 cells (Figure 3A) and was 10^5 times more effective than control Fab-SEA_{D227A}, as determined by the EC_{50} value. The EC_{50} was approximately 10^{-12} M for 5T4FabV13-SEA_{D227A} and no cytotoxicity was observed in the absence of T cells. 5T4FabV13-SEA_{D227A} was also tested for the ability to mediate MHC class II-dependent superantigen-mediated cytotoxicity against chromium-labelled Raji cells (Figure 3B). 5T4FabV13-SEA_{D227A} had about 100 times reduced MHC class II-dependent cytotoxicity compared to control Fab-SEA, as judged by the EC_{50} value. This reflects the lowered affinity to MHC class II by the D227A substitution in SEa. In this assay, the EC_{50} value of 5T4FabV13-SEA_{D227A} was approximately 10^{-10} M. 5T4FabV13-SEA_{D227A} had similar activity as the control Fab-SEA_{D227A}.

In vivo immune activation of 5T4FabV13-SEA_{D227A}

In order to quantify the systemic immune response by Fab-SEA_{D227A} relative to Fab-SEA proteins, we analysed the serum levels of a panel of cytokines in C57/B16 mice injected with the same dose Fab-SEA and 5T4FabV13-SEA_{D227A}. There is a correlation between the systemic cytokine levels and systemic toxicity for Fab-SEA constructs in both mice and humans (Dohlstien et al, 1995b; Alpaugh et al, 1998). Repeated injections of Fab-SEA resulted in strong production of IL-2, IFN-γ, TNF-α and IL-6 (Figure 4). Drastic reduction in the systemic levels of all tested cytokines was seen when comparing the effects of Fab-SEA with 5T4FabV13-SEA_{D227A}. A 10- to 100-fold reduction in the serum levels of IL-2 and IFN-γ and a 100 to 1000 reduced amount of

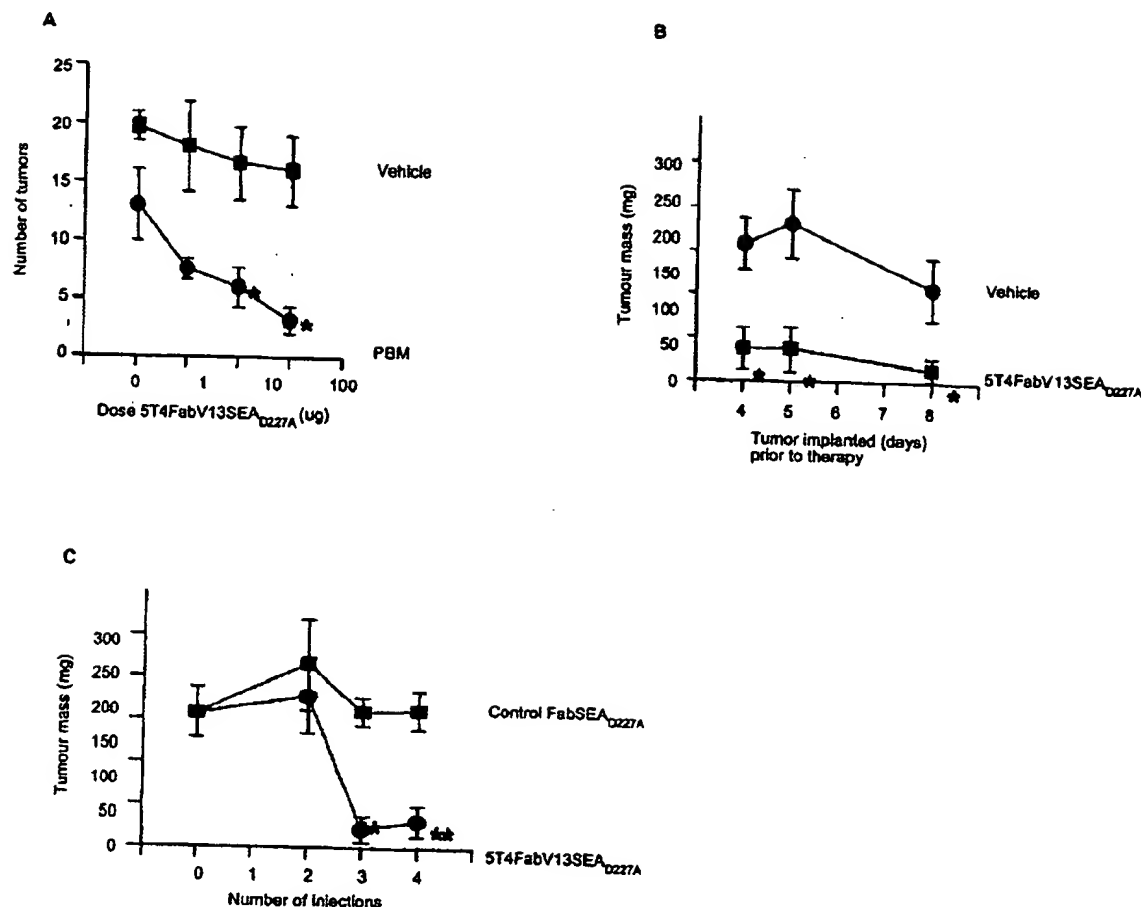


Figure 5 Therapy of SCID-mice carrying human Calu-1 tumours. (A) Dose titration, (B) effects of therapy on day 4, 5 and 8 tumours and (C) increased effects are observed using repeated injections of 5T4FabV13SEA_{D227A}. 5T4FabV13-SEA_{D227A} was active even on day 8 tumours, but only in the presence of PBM. Best effects were observed after repeated injections

TNF- α and IL-6 was recorded in animals treated with 5T4FabV13-SEA_{D227A} (Figure 4). In fact, the levels of IL-2 and TNF- α were below the level of detection for the assay using the superantigen analogue. Thus, the replacement in the MHC class II-binding region in SEA results in a drastic reduction in the systemic levels of cytokines in the murine system.

Therapy of human NSCLC with 5T4FabV13-SEA_{D227A} in humanized SCID mice

To investigate 5T4FabV13-SEA_{D227A} therapy against human NSCLC cells, a SCID-mouse model was developed. Calu-1 cells were tested for intraperitoneally growth in the mice (data not shown) and kinetic analysis showed an increased tumour growth during the first 30 days. All tumours were found to be 5T4 antigen positive using immunohistochemical staining 9, 10 and 25 days after transplantation. Using systemic intravenous treatment, 5T4FabV13-SEA_{D227A} was given 3 times with 3 day intervals of mice grafted with human PBMC, a strong suppression in Calu-1 tumour mass and number was obtained (Figure 5). More than 85%

reduction in the number of tumours and more than 95% reduction in tumour mass, calculated as described in Materials and methods, were observed. No significant therapy was observed with a control fusion protein not binding to the Calu-1 tumour, indicating that the therapy involves specific targeting to 5T4 positive tumour cells. Significant tumour therapy was only observed when treating with 5T4FabV13-SEA_{D227A} in the presence of PBMC (Figure 5). The 5T4FabV13-SEA_{D227A} therapy was dose-dependent requiring 10 µg/injection or more for a significant effect. Depending on the T-cell donor however, significant therapy can sometimes be seen with 1 µg/injection of 5T4FabV13-SEA_{D227A} (data not shown). 3 or more injections of 5T4FabV13-SEA_{D227A} were required for optimal tumour therapy in the humanised SCID mice (Figure 5). Significant therapy of Calu-1 growth was obtained against 4, 5- and 8-day-old established tumours (Figure 5). A more than 85% reduction of tumour weight was observed on 8-day-old established tumours.

Thus, significant dose-dependent tumour therapy against established NSCLC tumours growing in humanized SCID mice was obtained with 5T4FabV13-SEA_{D227A}.

DISCUSSION

NSCLC is associated with a poor prognosis since the beneficial effects of the available therapies are limited. Current therapeutic protocols include surgery and chemotherapy, but despite recent improvements most advanced stage patients die of the disease. However, it has clearly been shown that human non-small-cell lung cancer tissue contain tumour-infiltrating lymphocytes that upon activation releases tumoricidal cytokines (Ortega et al, 2000). Thus, the fusion protein 5T4FabV13-SEA_{D227A} represents a novel and attractive approach for therapy of NSCLC. The 5T4FabV13 has a high affinity for the antigen and can therefore be used for efficient targeting of superantigens to the tumour tissue. The SEA variant used has very potent T cell activating as well as cell killing properties and it has been modified to reduce systemic toxicity. Fusing the superantigen and 5T4FabV13 has not significantly altered their individual properties and the recombinant product can be produced at very high levels in *E. coli*, which is not always the case of recombinant antibody fragments (reviewed by Hudson, 1998). The favourable reactivity of the 5T4 antigen in all tested NSCLC and breast carcinomas in combination with the low normal tissue reactivity suggests that these types of cancer cells constitute good targets for the fusion protein. Also, the tumour stroma contained large amounts of the 5T4 antigen (Figure 1) and may therefore be an additional target for the fusion protein. Whether the binding of 5T4FabV13-SEA_{D227A} to stromal cells contributes in the eradication of solid tumours remains to be studied. Most of the normal tissue reactivity found was found to be weak and focal/diffuse. The reaction associated to the gastro-intestinal tract is the most prominent. However, the nature of this reactivity, whether it is cell-bound, cell surface associated or extracellular can not be concluded from light microscopy analysis and thus it is not possible to make predictions of 5T4FabV13-SEA_{D227A} targeting to these structures in vivo. This is also the case for the reaction seen in association to lung bronchial epithelium. In a previous clinical phase I study, no obvious organ-related side effects were seen in cancer patients using C242FabSEA (Alpaugh et al, 1998) which binds strongly to MHC class II as well as to normal colon tissue.

Treatment of certain neoplastic disease with monoclonal antibodies is effective. Very encouraging data has been presented for B-cell malignancies (McLaughlin et al, 1998), colorectal cancer (Riethmüller et al, 1998) as well as Her-2 positive breast cancer (Goldenberg, 1999). Traditionally murine antibodies were used, but more recently human or humanized antibodies have shown to have advantages. There is an intense focus on other antibodies in the preclinical or early clinical phase, but there are also activities ongoing to further potentiate the successful antibodies using radioisotopes, cytotoxic fusion partners or by making the antibodies bispecific. Targeting of superantigens has previously been described for colon cancer therapy (Dohlsten et al, 1994, 1995b). This therapy leads to infiltration of T-cells in the tumour tissue (Dohlsten et al, 1995a, Litton et al, 1996). Superantigen therapy stimulates both CD4+ and CD8+ T-cells (Dohlsten et al, 1995a) that have potent cytotoxic properties to directly kill target cells, but secondary effects such as cytokine secretion and recruitment of other effector cells may be of even higher importance for successful therapy. These secondary events are important to kill sub-populations of cancer cells not expressing the antigen. Targeting of wild-type SEA has been investigated in phase I clinical studies for colon cancer therapy (Alpaugh et al, 1998). Preclinical data also suggest that these already powerful molecules

can be further potentiated by the simultaneous targeting of IL-2 to the tumour tissue (Rosendahl et al, 1999; Soegaard et al, 1999). Studies using bispecific antibodies and staphylococcal enterotoxin B (Rice et al, 1999), show that this combination induce anti-tumour immunity and since 5T4FabV13-SEA_{D227A} uses similar immunological principles for therapy, it is tempting to speculate that also here a T-cell memory is induced.

The structure and function properties of SEA have been well characterized (Abrahmsén et al, 1995; Hudson et al, 1995; Schad et al, 1995). To reduce the systemic toxicity, a major drawback in the use of wild-type SEA (Alphaug et al, 1998), a substitution of Asp 227 has been made. This residue co-ordinates a Zn²⁺ ion to form a high affinity binding site to the β -chain of MHC class II. However, binding to MHC class II is important to obtain inflammatory cytokines and to activate T-cells. Therefore, the second MHC class II binding site surrounding Phe 47 was not altered. The biological consequences of this replacement is that the soluble fusion protein is less potent in activating resting T cells and stimulating cytokine production, due to a lower MHC class II affinity. Although the mouse is much less sensitive to SEA-induced immune activation, our data clearly demonstrates the relative potency difference in the mutated superantigen construct. This difference has been confirmed in other species such as the rabbit and monkey (data not shown), and supporting data are being obtained in humans. However, the fusion protein is equally very active when presented on a cell surface via the 5T4-antigen. Therefore, it is anticipated that the product will be very potent locally, e.g. in the tumour, while being less potent in systemic T-cell activation. Also, targeting to tissues such as the spleen is less pronounced with the mutated superantigen (Hansson et al, 1997).

One important attribute of SEA therapy is that it does not depend on the MHC-restricted T-cell recognition of peptide antigens where evasion of natural or induced CTLs may occur by down-regulation of HLA class I expression (Keating et al, 1995; Garidido et al, 1997). There are now several well-established examples of such immune escape in the natural history of neoplasia (Bontkes et al, 1998, 1999) and there is little doubt that this presents a major problem for cancer vaccine therapies aimed at inducing CTL responses.

In conclusion, 5T4FabV13-SEA_{D227A} combines excellent tumour cell-binding properties with the powerful cytotoxic properties carried by the superantigen and represents a novel type of therapy against non-small-cell lung cancer. The 5T4FabV13-SEA_{D227A} is currently investigated in a phase I clinical study in NSCLC patients.

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Mutations in the MHC Class II Binding Domains of Staphylococcal Enterotoxin A Differentially Affect T Cell Receptor V β Specificity¹

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C-terminal residues of staphylococcal enterotoxin A (SEA), including H187, D225, and D227, are involved in moderate affinity binding to MHC class II β -chain, whereas N-terminal residues, including F47, are involved in low affinity binding to MHC class II α -chain. The effect of alanine substitutions at residues D227 or F47 on induction of T cell proliferation and the expansion of specific TCR V β families was determined. SEA wild type specifically activated T cells expressing V β 1, V β 5.2, V β 6, V β 7, V β 9, V β 18, and V β 22. Although SEA-D227A exhibited substantially reduced mitogenicity compared with SEA wild type, it expanded the same V β -bearing T cells, except those expressing V β 1. By contrast, SEA-F47A, which was slightly less mitogenic than SEA wild type, induced expansion only of T cells expressing V β 6, V β 7, and to a lesser extent V β 22. Therefore, specific mutations affecting either MHC class II α or β binding sites differentially affect the V β specificity of this superantigen. The lack of expansion in four of seven V β families by SEA-F47A suggests that the class II α binding site may position SEA on the MHC class II molecules in an appropriate conformation for interaction with certain V β elements. *The Journal of Immunology*, 1996, 157: 3988–3994.

Superantigens are bacterial and viral products that are among the most potent activators of T cell proliferation and cytokine production and can be distinguished from conventional Ags by their ability to stimulate large numbers of T cells in a V β -specific manner (1–4). Unlike conventional Ags, superantigens do not require complex processing because they can bind directly to MHC class II molecules at residues outside the peptide binding groove and as a result are able to be “presented” to T cells by either syngeneic, allogeneic, or xenogeneic MHC class II molecules (5–8). Each superantigen has a characteristic affinity to a set of TCR V β elements and can interact with most T cells expressing those elements, potentially up to 20% of the total T cell population (1–4). Depending on the context of the interaction and the nature of costimulatory signals exchanged between T cells and APCs, superantigen-specific T cells may undergo either specific expansion, deletion, or anergy (reviewed in Ref. 9).

The ability of superantigens to bridge T cells and APCs and to utilize the TCR and MHC class II molecules as signaling receptors contributes greatly to their induction of potent immunologic responses not only by stimulating strong proliferation of T cells but also by concomitantly eliciting the secretion of excessive quantities of inflammatory cytokines (10). This superantigen-induced inflammatory response is thought to be responsible for the symptoms

associated with staphylococcal food poisoning as well as staphylococcal and streptococcal toxic shock syndromes (1, 9, 11–13). Superantigen-mediated immune responses have also been suspected to play a role in triggering or exacerbating certain autoimmune diseases (reviewed in 9, 12, 14, 15). In addition, superantigens have been shown to direct T cell-mediated killing of tumor cells (16). Understanding the various roles of superantigens requires elucidation of the structure/function relationship of these molecules as well as an explication of the molecular basis of their interaction with TCR and MHC class II molecules.

The staphylococcal enterotoxin family of superantigens (SEA, SEB, SEC₁₋₃, SED, and SEE) are considered prototypical superantigens and have been used in a variety of studies attempting to decipher mechanisms of superantigenicity. Recent analyses have identified a number of functional regions of staphylococcal enterotoxin A (SEA),² including domains involved in MHC class II binding (17–23), TCR recognition (20, 24, 25), and biologic activity (26–28). SEA mutants with site-directed substitutions in either the N-terminal class II α or the C-terminal class II β binding domains of SEA have been generated and characterized with respect to their MHC class II binding affinity (20–23). N-terminal residues, including F47A, are thought to be important in binding to the MHC class II α -chain, whereas C-terminal residues, including H187, D225 and D227, are believed to be involved in Zn-coordinated binding to the class II β -chain (21). Previous studies have shown that the mutants SEA-F47A and SEA-D227A exhibit an ~7-fold and >1000-fold higher K_d for class II than SEA wild type (wt), respectively (21). These data suggest that the N terminus of SEA forms a low affinity interaction with MHC class II, while the C terminus exhibits a moderate affinity binding; however, both regions are required for high affinity binding to MHC class II (21). The aim of this study was to investigate whether mutations in the low and moderate affinity binding sites alter the *in vitro* V β specificity of SEA. The results indicate that certain TCR V β families seem to recognize SEA independent of the topology of the

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³ Abbreviations used in this paper: SEA, Staphylococcal enterotoxin A; SEA-F47A, -N128A, -H187A, or -D227A, SEA with alanine substitutions at residues -F47, -N128, -H187, or -D227; TCC, human T cell clone; wt, wild type.

superantigen/MHC complex, while the interaction with other V β elements may require more rigid fixation of SEA to the α -chain of MHC class II molecules.

Materials and Methods

Wild-type and mutant SEA

Recombinant SEA wt was expressed in *Escherichia coli* as previously described (21). Briefly, *E. coli* K12 strain UL635 was transformed with plasmid pKP943 containing the gene for SEA, the constitutive promoter from staphylococcal protein A, and a synthetic signal peptide. SEA mutants (SEA-F47A, SEA-N128A, and SEA-D227A) were generated by site-directed mutagenesis using PCR and cloned in *E. coli* K12 strain HB101 as previously described (21). Host cells were grown overnight at 24 to 34°C in 2X YT (16 g/L Bacto tryptone, Difco Laboratories, Detroit, MI; 10 g/L Bacto yeast extract, Difco; 5 g/L NaCl, Sigma Chemical Co., St. Louis, MO) supplemented with kanamycin (50 mg/ml). Cells were pelleted at 5000 \times g, and the periplasmic contents were released by 1 cycle of gentle freeze-thawing in 10 mM Tris-HCl (pH 7.5). SEA wt and mutant SEA proteins were immunoaffinity purified from periplasmic lysates using polyclonal rabbit anti-SEA Abs coupled to 5-ml NHS-activated HiTrap columns (Pharmacia Biotech, Lund, Sweden), and the immunoaffinity fraction was further purified by HPLC. SEA wt and mutants were all found to be >95% pure when analyzed by SDS-PAGE.

Induction of proliferation by SEA wt and mutants

PBMCs obtained from healthy donors were separated from whole heparinized blood by centrifugation over a Ficoll/Hypaque gradient. Cells (1×10^5 /well) were cultured in triplicate in complete medium (RPMI 1640, Life Technologies, Gaithersburg, MD; 25 mM HEPES, Life Technologies; 0.1 mg/ml gentamicin sulfate, Biologic Industries, Kibbutz Beit Haemek, Israel; 4 mM L-glutamine, Life Technologies; 10% heat-inactivated fetal bovine serum, Atlanta Biologicals, Norcross, GA) and stimulated with SEA wt or SEA mutants. Cultures were incubated at 37°C, 5% CO $_2$, and 95% humidity for 5 days and then pulsed with 1 μ Ci of [3 H]thymidine (sp. act. 6.7 Ci/mmol, NEN-DuPont, Wilmington, DE) 4 h before harvest. Cells were harvested onto glass fiber filters, and [3 H]thymidine incorporation was determined by a Packard liquid scintillation counter. Each culture condition was performed in triplicate wells, and all experiments included PHA (1 μ g/ml, Sigma) or Con A (1 μ g/ml, Sigma) and cells alone to serve as positive and negative controls, respectively.

In vitro T cell stimulations with SEA

PBMCs, separated from whole blood of healthy donors by centrifugation over Ficoll/Hypaque, were subjected to 1 cycle of erythrocyte rosetting. Enriched T cells were cultured (1.5×10^4 cells/ml) at 37°C in a humidified atmosphere containing 5% CO $_2$ in the presence of SEA wt (10 nM), SEA-D227A (1 μ M), SEA-N128A (10 nM), or SEA-F47A (100 nM) for 3 days. Parallel control cultures were stimulated with either anti-CD3 mAb (OKT3, 0.1 μ g/ml) or the polyclonal mitogen PHA (5 μ g/ml). Viable cells were recovered from the cultures on day 3 by centrifugation over Ficoll/Hypaque and cultured for an additional day in complete medium supplemented with 10 U/ml recombinant human IL-2. In some experiments, CD4 and CD8 T cells were separated at the end of the culture period using negative and positive selection with Dynal M450 magnetic beads (Dynal, Inc., Lake Success, NY). After separation, positively selected cell-bound beads were removed using the Detach-a-Bead method (Dynal) according to the manufacturer's protocol. To assess the quality of separation, cells from each fraction were analyzed by flow cytometry using a FACStarPlus flow cytometer (Becton Dickinson, Mountain View, CA). Positively selected cells were reciprocally stained with anti-CD4-FITC or anti-CD8-PE and were found to be >98% pure (data not shown).

Analysis of TCR V β repertoire

Analysis of preferential V β expansion was conducted by reverse transcription (RT)-PCR as previously described (29). Total RNA was extracted from cells cultured with SEA wt or mutants using RNeasy (Qiagen, Crawfordsville, TX) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using 2–5 μ g of total RNA utilizing random hexamers as primers (pdN6, Pharmacia Biotech, Inc., Piscataway, NJ) and Superscript reverse transcriptase (200 U/mg RNA, Life Technologies). Before amplification, the cDNA was titrated in a pilot experiment to determine the appropriate amount required for linear amplification. The cDNA was aliquoted into 26 tubes and amplified in the presence of V β family-specific 5' primers and a 3' C β primer. As an internal control, each tube contained 5' forward and 3' reverse C α primers to generate a 600-bp product

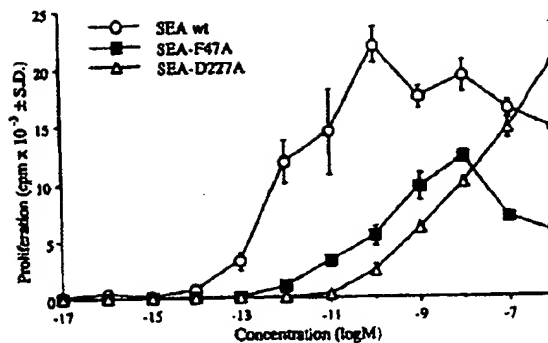


FIGURE 1. In vitro proliferative response induced by SEA wt and mutants. PBMCs from a healthy donor were cultured (1×10^5 cells/well) with increasing dilutions of SEA wt (open circle), SEA-F47A (closed square), and SEA-D227A (open triangle) for 5 days at 37°C and 5% CO $_2$. Each well was pulsed with 1 μ Ci [3 H]thymidine 4 h before the cultures were harvested. Data are presented as mean cpm \pm SD of triplicate wells from a representative experiment that was repeated with four donors.

(all primers were used at 0.3 μ M). The reaction mixture included 50 μ l of 1 \times PCR buffer (50 mM KCl; 20 mM Tris-HCl, pH 8.4; 1.5 mM MgCl $_2$; 0.1 mg/ml nuclease-free BSA) plus 100 μ M deoxyribonucleoside triphosphate mix and 1.25 U Taq DNA polymerase. To allow for quantitation, 10^5 cpm of [3 P]-labeled 3' C α and C β primers were included in each tube. The primers were end-labeled using T4 polynucleotide kinase (Promega, Madison, WI) for 1 h at 37°C with 250 μ Ci of γ - 32 P-dATP (sp. act. 6000 Ci/mmol, NEN-DuPont) in each reaction. Unincorporated labeled nucleotides were removed using Sephadex G-25 spin columns (Boehringer Mannheim, Indianapolis, IN). Amplification occurred for 20 to 25 cycles of 1 min denaturation at 95°C, 1 min annealing at 60°C, and 1.5 min extension at 72°C. Radiolabeled products were separated on 2% agarose gels and exposed to x-ray films. Autoradiograms were scanned, and the relevant bands were quantified using a Discovery Series Model DNA 35 white light scanner and Quantity One software (PDI, Inc., New York, NY). PCR values for the V β bands were normalized by dividing the area value of each V β band by the area value of the coamplified C α band. To determine whether a particular V β was expanded, the following ratio was obtained: normalized PCR value V β (experimental)/normalized PCR value V β (anti-CD3 or PHA). A ratio >1 indicated expansion of the particular V β . This method has been used extensively in our lab and has been shown to correlate well with flow cytometric analysis of V β repertoire (30).

Stimulation of human T cell clones

The human T cell clones (TCCs) used represent a variety of alloreactive, HLA class II-specific or Ag-specific, HLA class II-restricted T cells and were all CD4 (31). The assays to measure TCC proliferative responses to SEA wt and mutants was performed using TCC thawed the same day cultured with B-LCL cells from donor RN (DR3, DQ2 homozygous) as APCs. T cells (2.5×10^4) and irradiated B-LCL cells (1×10^4), in RPMI 1640 with 15% heat-inactivated human serum, were cultured in the presence of SEA wt, SEA-F47A, and SEA-D227A at the indicated concentrations. Stimulation with PHA (1 μ g/ml, Sigma) and recombinant human IL-2 (5 U/ml, Amersham International, Amersham, U.K.) in the presence of APCs served as a positive control. [3 H]thymidine was added after 48 h of incubation, and the cultures were harvested 24 h later.

Results

T cell proliferation induced by SEA wt and mutant proteins

Alanine substitutions at residues F47, N128, and D227 of SEA were generated by site-directed mutagenesis, and the PCR products were cloned and expressed in *E. coli* (21). To determine whether these mutations affect SEA-induced proliferation, a dose-response test of SEA wt and each mutant was performed with human PBMCs. The N-terminal mutant SEA-F47A and the C-terminal mutant SEA-D227A were 10^2 - and 10^4 -fold less potent than SEA wt, respectively (Fig. 1). By contrast, mutating residue N128, which forms a hydrogen bond with D227 and may stabilize its interaction with class II β -chain

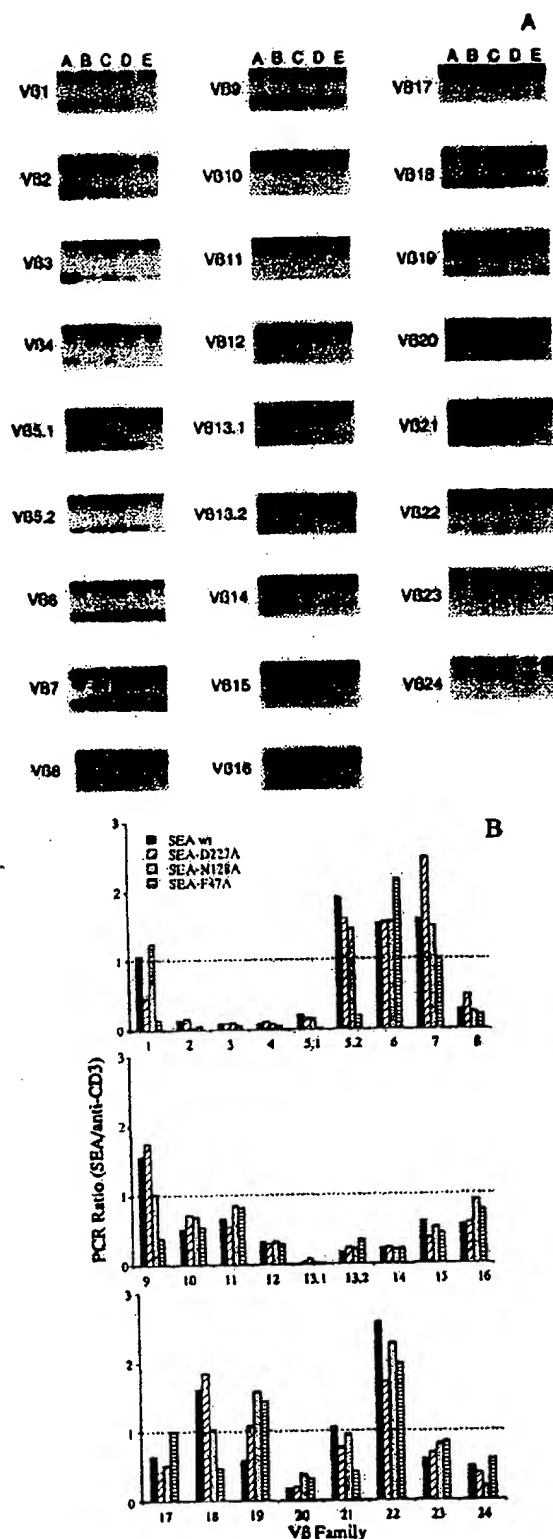


FIGURE 2. V β specificity of SEA wt and mutants. T cells were enriched with PBMCs from a healthy donor and cultured with SEA wt (10 nM), SEA-N128A (10 nM), SEA-D227A (1 μ M), SEA-F47A (100 nM), or OKT3 (0.1 μ g/ml) for 3 days. V β analysis was performed by RT-PCR as described in Materials and Methods with 10^5 cpm 32 P-labeled 3'-C α -

residues, had an insignificant effect on the mitogenicity of the protein (data not shown). As expected, the double mutant SEA-F47A/D227A induced only a low response and was less than 10^6 -fold as potent as SEA wt (data not shown). Inasmuch as previous studies have shown that the affinity of SEA-F47A and SEA-D227A to MHC class II is 5–10- and >1000-fold less than SEA wt, respectively (21, 22), our data indicate that a mutation in the low affinity class II α binding domain of SEA has much less of an impact on the proliferative response than a mutation in the moderate affinity class II β binding domain.

In vitro TCR V β specificity of SEA wt and mutants

The *in vitro* V β specificities of SEA wt, SEA-F47A, SEA-N128A, and SEA-D227A were compared. Human T cell-enriched populations were cultured with optimal concentrations of the various SEA proteins or a polyclonal T cell stimulator (anti-CD3 or PHA). The expression of V β families was analyzed by RT-PCR, and V β -specific expansion was assessed by comparing expression in cultures with superantigens to those with polyclonal mitogens. Figure 2 shows the entire V β spectrogram from a representative individual, and Figure 3 shows the pattern of expansion of the relevant V β s from stimulations performed with T cells from different individuals. Although there is some interindividual variation in the level of V β expansion, the pattern was consistent among the series of analyzed healthy donors. SEA wt caused expansion of V β 1, V β 5.2, V β 6, V β 7, V β 9, V β 18, and V β 22, with V β 19 being expanded in cells from two of four individuals examined. A similar pattern was seen in SEA-D227A-stimulated cells, except that this C-terminal mutant failed to stimulate V β 1-bearing T cells. By contrast, the V β specificity of SEA-F47A was drastically different from SEA wt, causing expansion of only V β 6- and V β 7-bearing T cells and marginal expansion of V β 22 in most individuals studied. The mutation in residue N128 did not alter the *in vitro* V β specificity compared with SEA wt (Fig. 3). It is noteworthy that none of the SEA mutants studied here caused expansion of any V β that failed to respond to the wild-type protein; rather, they were unable to stimulate T cells bearing relevant V β s.

SEA-Induced activation of human T cell clones

To further investigate the V β specificity of SEA wt and mutant proteins, we examined their ability to induce proliferation of human T cell clones expressing defined V β elements. Human T cell clones expressing either V β 1.1, V β 1.2, V β 5.7 (which is also amplified by the V β 5.2 PCR primer used here), V β 6.7c, or V β 7.1 were assessed for their ability to proliferate in response to SEA wt, SEA-F47A, or SEA-D227A, presented by fixed MHC class II-expressing EBV-transformed B cells. As shown in Table I, SEA wt and SEA-D227A stimulated all five clones to a similar extent, whereas SEA-F47A stimulated only the clones expressing V β 6.7c and V β 7.1 elements. Thus, the overall pattern of specificity was in agreement with that determined using the RT-PCR technique. The fact that SEA-D227A stimulated V β 1.1 and V β 1.2 clones and was more potent than SEA wt in stimulating the V β 7.1 clone may be

and 3'-C β primers included in each reaction. PCR products were separated on a 2% agarose gel that was then dried and exposed to x-ray film. An autoradiogram from a representative individual is shown in A: lane A, OKT3; lane B, SEA wt; lane C, SEA-D227A; lane D, SEA-N128A; and lane E, SEA-F47A. Autoradiograms were scanned to determine band intensity, and PCR values were calculated as follows: (SEA V β /SEA C α)/[OKT3 V β /OKT3 C α]. PCR values >1 indicate specific V β expansion. B, PCR values obtained from the scanned bands shown in A.

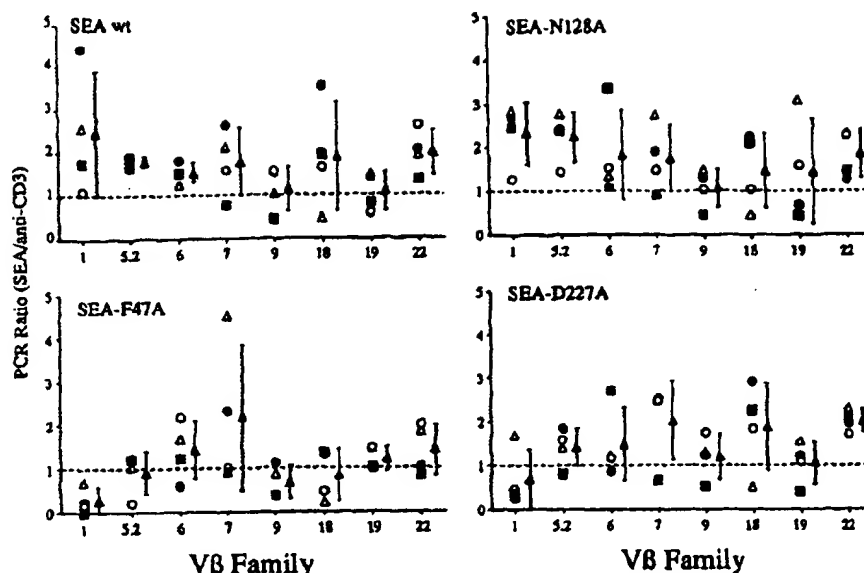


FIGURE 3. PCR values of SEA-specific V β s from different individuals. Enriched T cells from different individuals were each stimulated with SEA wt or the indicated SEA mutant proteins. Analysis of V β expansion was performed by RT-PCR as described in the legend for Figure 2. Each symbol represents data from an individual, and only the V β s relevant to SEA are shown. The mean PCR value \pm SD for each V β is indicated by a closed triangle.

Table 1. Proliferative response of human T cell clones to SEA wt and mutants^a

| T Cell Clone | Stimulation Index ^b | | |
|------------------------|--------------------------------|-----------|----------|
| | SEA wt | SEA-D227A | SEA-F47A |
| IW 19 (V β 1.1) | 4.0 | 5.1 | 2.7 |
| MW4.66 (V β 1.2) | 15.7 | 11.1 | 2.4 |
| RT-1 (V β 5.7) | 4.3 | 4.1 | 1.6 |
| OB44 (V β 6.7c) | 28.0 | 25.6 | 22.0 |
| RT-3 (V β 7.1) | 7.8 | 15.6 | 4.0 |
| IW 17 (V β 7.1) | 36.0 | 22.0 | 8.7 |

^a Clones were cultured with SEA wt (10 nM), SEA-D227A (100 nM), or SEA-F47A (100 nM) in the presence of fixed EBV-transformed B cells. After 2 days each well was pulsed with 1 μ Ci [³H]thymidine 24 h before the cultures were harvested.

^b Stimulation index = cpm of experimental culture/background cpm of unstimulated cells. The background cpm for each clone was as follows: IW 19, 3500 cpm; MW4.66, 500 cpm; RT-1, 2000 cpm; OB44, 500 cpm; RT-3, 500 cpm; and IW 17, 188 cpm. Data are from a representative experiment of three performed.

specific for these clones and may reflect, as previously suggested (32, 33), an influence of the V α -chain on the interaction between this mutated superantigen and the TCR.

Analysis of V β expression by CD4 and CD8 T cells stimulated with SEA wt and mutants

To determine whether SEA wt and the SEA mutants induce different levels of V β expansion in CD4 and CD8 T cell subsets, T-enriched cell cultures were stimulated with SEA wt or the various mutants and then separated into CD4 and CD8 cells using magnetic beads. The TCR V β repertoire was analyzed in the CD4 and CD8 populations by RT-PCR. SEA wt expanded T cells bearing V β 1, V β 5.2, and V β 6 to a similar extent in both the CD4 and CD8 populations, whereas SEA-D227A induced slight expan-

sion of V β 1-, V β 5.2-, and V β 9-bearing T cells only in the CD4 population (Fig. 4). Both SEA wt and SEA-D227A caused expansion of V β 7, V β 9, and V β 18 to a greater extent in CD4 compared with CD8 T cells. SEA-F47A expanded V β 6, V β 7, and V β 22 to a similar extent in both CD4 and CD8 T cells. Fractionation of cells also uncovered an additional, apparently weakly reactive, interaction with V β 16 CD4 T cells by SEA wt and mutants. The effects of each mutation on V β -specific expansion are summarized in Table II.

Discussion

SEA is considered to be one of the strongest naturally occurring MHC class II ligands and has been shown to have two MHC class II binding domains: a moderate affinity binding site located at the C-terminal region that includes residues H187, D225, and D227, which form a Zn²⁺ coordination site presumably involved in binding to residue H81 on the β -chain of the MHC class II molecule (2, 21, 34–36), and a low affinity site located at the N-terminal region of SEA resembling a site found in SEB that is believed to be involved in binding to the MHC class II α -chain (21, 37–39). High affinity binding to class II involves residues in both the C- and N-terminal regions of SEA (21, 22).

Previous studies have shown that although superantigens can interact directly with certain TCR V β elements in an MHC class II-independent manner (40–45), such interaction does not result in functional activation of T cells, which require costimulatory signals delivered via the CD28 pathway (42, 43, 45, 46). Accordingly, we have previously proposed that the ability of superantigens to simultaneously bind both TCR and MHC class II molecules targets professional APCs to T cells (14, 45). This allows efficient interaction of costimulatory molecules with their respective ligands in delivery of biochemical signals required for activation of T cells and APCs (14, 45). However, these studies did not address the structural constraints required for orientation of the superantigen in

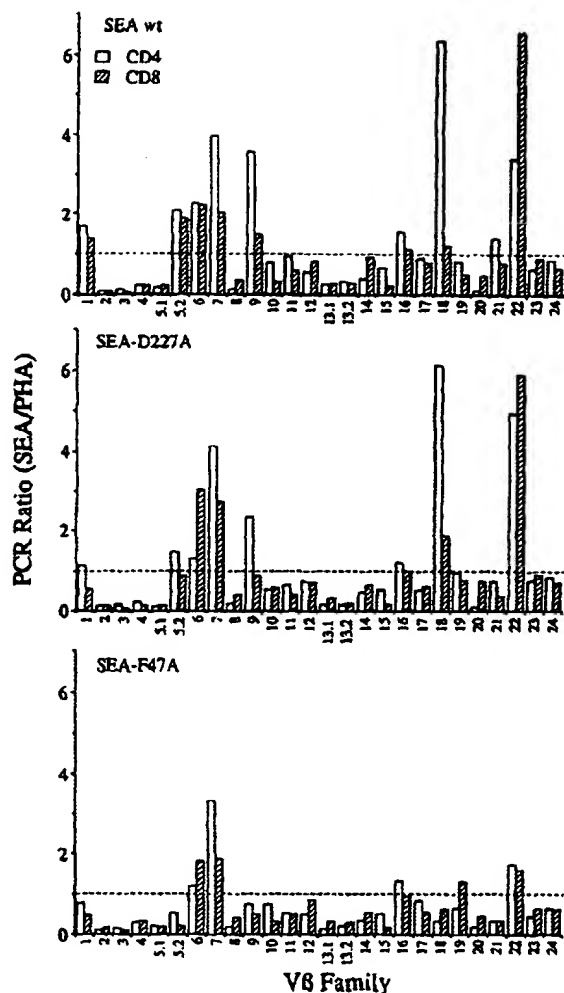


FIGURE 4. SEA-specific V β expansion in CD4 and CD8 T cells. Enriched T cells were stimulated with SEA wt (10 nM), SEA-D227A (1 μ M), SEA-F47A (100 nM), or PHA (5 μ g/ml). After 3 days in culture, the cells were separated into CD4 (open bars) or CD8 (hatched bars) populations by magnetic beads, and TCR V β analysis was performed by RT-PCR. Data are the PCR values from a representative experiment performed with cells from three individuals. A PCR value >1 indicates V β -specific expansion.

a way that allows proper interaction of superantigen/TCR/MHC complex as well as the optimal interaction of the costimulatory machinery.

In this study we examined the effect of mutations in the moderate and low affinity class II binding regions of SEA on the interaction of SEA with specific TCR V β elements. SEA wt caused expansion of V β 1-, V β 5.2-, V β 6-, V β 7-, V β 9-, V β 18-, and V β 22-expressing T cells, a pattern that corresponds well with the previously reported V β profile of this superantigen (20, 22). Mutations in the moderate affinity class II binding site, represented by SEA-D227A, which binds to class II with >1000-fold less affinity compared with SEA wt (21, 22), resulted in a dramatically reduced proliferative response. However, this mutation had little effect on the TCR V β specificity of the molecule. Using RT-PCR analysis

Table II. Summary of V β specificity of SEA wt and mutants

| Toxin | V β Expanded* | | | | | | | |
|-----------|---------------------|-----|---|---|---|----|------|------|
| SEA wt | 1 | 5.2 | 6 | 7 | 9 | 18 | (19) | 22 |
| SEA-N128A | 1 | 5.2 | 6 | 7 | 9 | 18 | (19) | 22 |
| SEA-D227A | | 5.2 | 6 | 7 | 9 | 18 | (19) | 22 |
| SEA-F47A | | | 6 | 7 | | | (19) | (22) |

*V β s in parentheses indicate that V β was not expanded in all individuals studied.

of V β expansion or evaluating the proliferative response of human T cell clones with defined V β specificities, we demonstrated that the pattern of V β expansion elicited by SEA-D227A was quite similar to that found in SEA wt-stimulated cells. These data are in agreement with those of Hudson et al. (22), who demonstrated that a mutation in another residue involved in Zn²⁺ coordination (H187) also had little effect on the TCR V β specificity of the molecule.

Although a mutation in the low affinity N-terminal binding region had relatively less effect on induction of proliferation compared with the C-terminal mutation, it had a profound effect on the TCR V β specificity of the molecule. Mutant SEA-F47A, which binds to MHC class II with 5- to 10-fold less affinity than SEA wt (21), induced a plateau in proliferation of PBMCs at 50% of the level as SEA wt at a saturating concentration of 10 nM, while SEA-D227A did not reach plateau levels at this concentration. The lower plateau level of SEA-F47A compared with SEA wt suggested that only a fraction of the CD4 and CD8 T cells were responding. Surprisingly, RT-PCR analyses revealed that SEA-F47A lost its ability to interact with V β 1-, V β 5.2-, V β 9-, and V β 18-bearing T cells. This mutant was capable of stimulating only V β 6-, V β 7-, and to a lesser extent V β 22-bearing T cells. The reason for the loss of V β specificity in the SEA-F47A mutant is not entirely clear because F47 has not been identified as a TCR-binding residue and because it is distantly located from the putative TCR groove on the deduced SEA structure (1, 20, 25, 36, 47, 48). However, this residue has been suggested to be involved in the binding of SEA to MHC class II α -chains (21). It appears, therefore, that binding to MHC class II α -chain may orient SEA for proper interaction with certain TCR V β elements. Another possibility, which is not mutually exclusive, is that a mutation at residue F47 may cause a conformational change that alters the binding of SEA to certain V β elements.

Our data suggest that not all V β elements are affected by mutations in the MHC class II binding domains. This may be related to differences in the affinity between certain V β elements and SEA or to differences in structural constraints for functional binding of MHC class II, SEA, and TCR. Interaction of SEA with V β 6 and V β 7 seems to occur independent of MHC class II binding (D. Newton, manuscript in preparation). By contrast, mutations affecting either the interaction with MHC class II α - or β -chains abolished the ability of the superantigen to stimulate V β 1-bearing resting T cells. It is possible that activation of V β 1 cells by SEA requires bivalent binding, possibly involving class II cross linking, for transduction of efficient signals required for T cell activation. Although SEA-D227A failed to stimulate V β 1-bearing, unfractionated resting T cells, it caused slight expansion of V β 1-expressing CD4 T cells and was as effective as SEA wt in stimulating V β 1.1- and V β 1.2-bearing T cell clones. The reason for this discrepancy is not entirely clear; however, Lundin et al. (31) have shown that certain T cell clones expressing identical V β elements respond differently to the same staphylococcal enterotoxins. This

effect can be explained by the fact that certain TCR V α residues (33), as well as MHC class II polymorphism (49), can affect V β interactions with superantigens, especially in the case of weakly reactive V β elements (32). It is possible that CD4 interaction with MHC class II may contribute to the stability of interaction between SEA and certain V β elements. An alternative, but not mutually exclusive, explanation is that resting T cells and T cell clones may have different signal requirements for activation such that the requirement for bivalent binding to class II to stimulate certain V β -bearing cells may not be needed for activation of T cell clones.

Although superantigens can form binary complexes with TCR or MHC alone, recent studies have demonstrated that the formation of trimeric TCR/superantigen/MHC complexes were more stable than either TCR/superantigen or superantigen/MHC binary complexes alone (44, 50). Initial hypotheses indicated that one SEA molecule was able to cross-link two MHC molecules, forming SEA₂MHC₂ complexes that may enhance signal transduction (21). However, SEA₂MHC₁ trimers have also recently been identified by isoelectric focusing gel electrophoresis, even under conditions where MHC molecules were at a molar excess (51). Regardless of the stoichiometry of the SEA/MHC complex, our data suggest that the low affinity class II binding site, which involves residue F47, may have a strong influence on the stability of interactions with certain V β elements. The dissection of the magnitude of the proliferative response and the number of V β elements that can interact with SEA suggests that for certain TCR V β elements, the orientation of superantigens as well as the binding affinity between superantigen and MHC class II and/or TCR is important for activation. It appears that the interaction of SEA with MHC class II α -chains may position the superantigen in the proper orientation for interaction with certain V β elements, while the interaction of superantigen with the β -chain of MHC class II may be more important for the delivery of activation signals required for optimal communication between APCs and T cells. A comparison of SEA-F47A-sensitive TCR V β -chains (such as V β 1, V β 5.2, and V β 18) and SEA-F47A-insensitive TCR V β -chains (such as V β 6 and V β 7) in structural studies may contribute to unraveling the molecular basis of superantigen interaction with immune recognition receptors.

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03-25691

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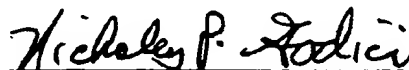
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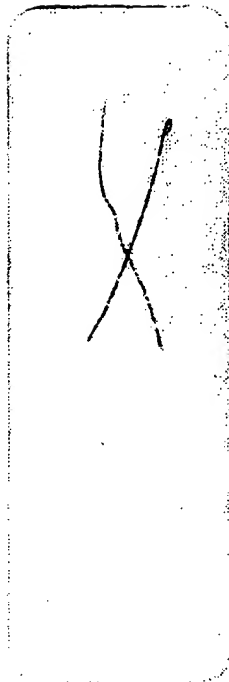
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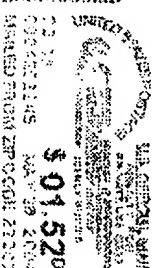
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